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Relaxation of Selection With Equalization of Parental Contributions in Conservation Programs: An Experimental Test With *Drosophila melanogaster*

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ABSTRACT

Equalization of parental contributions is one of the most simple and widely recognized methods to maintain genetic diversity in conservation programs, as it halves the rate of increase in inbreeding and genetic drift. It has, however, the negative side effect of implying a reduced intensity of natural selection so that deleterious genes are less efficiently removed from the population with possible negative consequences on the reproductive capacity of the individuals. Theoretical results suggest that the lower fitness resulting from equalization of family sizes relative to that for free contribution schemes is expected to be substantial only for relatively large population sizes and after many generations. We present a long-term experiment with *Drosophila melanogaster*, comparing the fitness performance of lines maintained with equalization of contributions (EC) and others maintained with no management (NM), allowing for free matings and contributions from parents. Two (five) replicates of size $N = 100$ (20) individuals of each type of line were maintained for 38 generations. As expected, EC lines retained higher gene diversity and allelic richness for four microsatellite markers and a higher heritability for sternopleural bristle number. Measures of life-history traits, such as egg-to-adult viability, mating success, and global fitness declined with generations, but no significant differences were observed between EC and NM lines. Our results, therefore, provide no evidence to suggest that equalization of family sizes entails a disadvantage on the reproductive capacity of conserved populations in comparison with no management procedures, even after long periods of captivity.

ONE of the main objectives of conservation programs of species in captivity is to preserve genetic variation to guarantee the evolutionary potential of populations and to avoid inbreeding, as it affects the reproductive capability of individuals (Frankham *et al.* 2002). As most captive populations are maintained with low census sizes, genetic drift is the main agent eroding genetic variation, and a number of procedures are recommended to mitigate its effects. One of the most simple and widely recognized methods is to equalize individual contributions, where each one of the individuals contributes exactly the same number of progeny to the next generation. This produces rates of inbreeding and drift that are about half those that would occur with free contributions from parents in an ideal population (Wright 1938; Gowe *et al.* 1959; Wang 1997a; Sánchez *et al.* 2003). More sophisticated methods, such as those looking for contributions of minimal coancestry, can be applied when pedigree or marker information is available and may give more efficient results (Ballou and Lacy 1995; Montgomery *et al.* 1997; Caballero and Toro 2000; Fernández and Caballero 2001b; Fernández *et al.* 2003b, 2004) although, in the absence of this information, equalization of contributions is the most appropriate procedure.

Equalization of contributions has the side effect of reducing the intensity of selection to about one-half (Haldane 1924; Hill *et al.* 1996). Because contributions from parents are intended to be homogeneous, selection between families is precluded (except by nonfertile couples) and only within-family selection operates. This has both positive and negative *a priori* consequences. On the positive side, because selection is minimized, adaptation to captivity is also reduced (Allendorf 1993; Frankham *et al.* 2000), and this is something desirable when the final objective of a conservation program is the reintroduction of the captive population into the wild (Loebel *et al.* 1992; Borlase *et al.* 1993; Couvet and Ronfort 1994; Frankham *et al.* 2000). On the negative side, the reduced purifying selection implies that many deleterious genes segregating in the base population and new mutations appearing during the program will more likely be fixed, with the corresponding consequences on the reproductive capacity of the individuals (see Bryant and Reed 1999). Therefore, one could argue that standard practices in

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conservation programs maximize the rate of accumulation of such alleles (Lange 1981; Couvet and Ronfort 1994); it is not immediately apparent that the benefits of preserving diversity by a particular program are not offset by such negative side effects.

Some theoretical studies have addressed the possible effects of the accumulation of deleterious alleles in conservation programs where contributions are equalized (Schoen *et al.* 1998; Fernández and Caballero 2001a,b; Theodorou and Couvet 2003). The conclusion from these studies is that equalization of contributions is expected to produce a higher fitness than random contributions in the short–medium term (up to ~10–20 generations). However, for longer periods, equalization of contributions may produce lower fitness than random contributions, particularly for large population sizes, although this difference can be very small depending on the mutational model assumed.

Considerable declines in fitness have been observed in lines subject to equalization of parental contributions for a substantial number of generations (Gilligan *et al.* 1997; Shabalina *et al.* 1997), the decline being ascribed to accumulation of mutations (Shabalina *et al.* 1997; but see Keightley *et al.* 1998 and Woodworth *et al.* 2002) or to inbreeding depression or adaptation to captivity (Gilligan *et al.* 1997). In these experiments, however, no comparisons were carried out between equalization of parental contributions and free contributions lines. This specific comparison has been examined only in one previous experiment (Borlase *et al.* 1993). They maintained lines of *Drosophila melanogaster* with four pairs of parents ($N = 8$) and two types of treatments (10 replicates of each) for 11 generations: lines where equalization of family sizes was achieved (each couple contributed one offspring of each sex to the next generation) and lines where contributions depended on the pairs offspring production. In this latter case, individuals were mated in pairs in single vials and relative family sizes were estimated from pupal numbers and by using random numbers to choose parents from the four available matings. It was found that, as expected, equalization of contributions produced a lower rate of increase in inbreeding and a higher average heterozygosity for allozymic markers than random contributions. Moreover, after 11 generations, fitness under equalization of contributions was about twice that of fitness under random contributions from parents. Thus, a clear significant advantage of equalization of contributions was inferred from this experiment.

Other related experiments were performed by Loebel *et al.* (1992) and Montgomery *et al.* (1997). In these cases, methods similar to equalization of contributions but more elaborate (equalization of founder representations and minimum kinship, respectively) were used and compared to random contributions. In both experiments a small number of individuals were used ($N = 8$) for a few generations (nine and four, respectively). No significant differences were found between the fitness of management and no management lines at the end of the experiments but, in the experiment of Montgomery *et al.* (1997), fitness with minimum kinship was always greater than or equal to that with random contributions.

Previous experiments suggest, therefore, that equalization of contributions does not imply a reduction in fitness and might even produce a greater fitness, relative to a scheme with random contributions. We should note, however, two issues related to the theoretical conclusions stated above. First, both the low population size used and the short number of generations considered in the experiments favor situations where equalization of contributions may outperform a no management scheme. Larger population sizes and longer generation periods may reverse the situation. Second, the establishment of random contributions in these experiments was done with independent monogamous matings, without allowing for competition among the parents and among full-siblings from different families. In a fully unmanaged scheme, purging selection from competition between individuals is expected to reduce the putative fitness advantage of equalization of contributions. Thus, to test a situation that may generate the most extreme differences between equalization of contributions and no management, this latter experiment should be performed by allowing for competition among parents and among offspring from different families, and the comparison should be made for sufficiently large population sizes and sufficiently long periods of time. In this article, we present the results of a long-term experiment (a total of 38 generations) with the model species *D. melanogaster* that compares the fitness of replicate lines maintained by equalizing the contributions of parents with the fitness of lines maintained with free mating and free contributions of parents under competitive conditions.

MATERIALS AND METHODS

Base population and culture conditions: A total of 546 inseminated females were collected in a wine cellar close to Vigo (northwest Spain) in October 2001. This wild population has been analyzed in previous studies (Fernández *et al.* 2003a; Rodríguez-Ramilo *et al.* 2004), showing normal levels of variation for egg-to-adult viability and fecundity. No substantial polymorphisms for inversions were found in a chromosome screening of the population (B. Fernández, personal communication). Flies were reared in a culture medium composed of 1 liter water, 200 g brewer's yeast, 50 g sucrose, 12 g agar, 2.5 g NaCl, and 5 ml propionic acid. A poorer medium with half as much yeast content (but twice the sugar) was used for the maintenance of the base population and for the fitness evaluation at generation 38. All cultures were maintained under continuous lighting in a chamber kept at a constant temperature of 25 ± 1° and relative humidity of 65 ± 5%. Flies were handled at room temperature under CO_2 anesthesia. Virgin males and females were used for matings across the entire experiment.

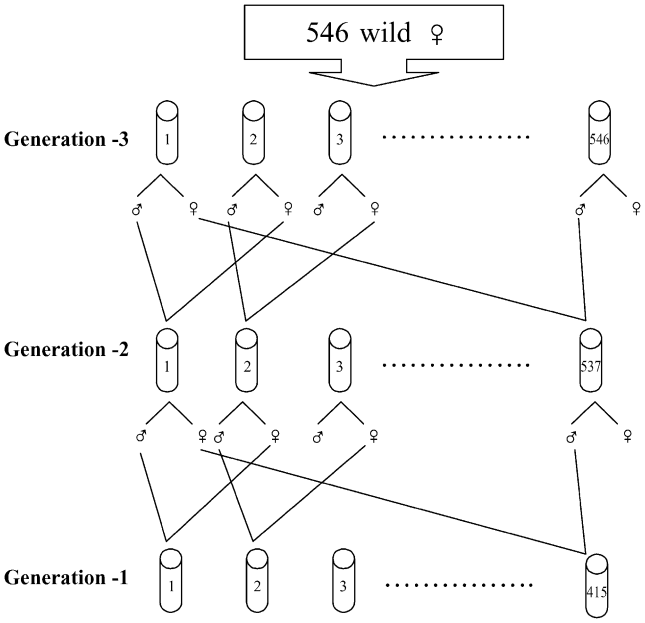

FIGURE 1.—Mating scheme prior to the start of the experiment.

Experimental lines: Prior to the start of the experiment, flies were maintained by single-pair matings in vials (20 mm diameter, 100 mm height with 5 ml of culture medium) following a circular scheme (Figure 1). This was carried out to minimize cross-generation maternal effects from wild flies that could produce artifactual differences between the experimental lines. However, the number of generations was reduced to a minimum because the objective was to start the experiment from a base as close as possible to the natural, recently caught, population. From those wild females (generation −3) producing at least one offspring of each sex, matings were established for two generations. From the 415 vials in generation −1 producing at least two progeny of each sex, 300 were chosen at random to constitute the basis of the experimental lines (Figure 2). Two male or two female progeny were chosen per vial to produce the replicate lines for each treatment. In the lines with no management (NM lines) individuals were maintained in bottles (of 500 ml with 100 ml culture medium), allowing for free mating and competitive contributions from parents to the next generation. In the lines of equalization of contributions (EC lines) pairs of individuals were crossed in vials, choosing one male and one female progeny at random. Mating in this case was established randomly except that pairing between full-sibs was avoided, as this is a usual procedure advised for the maintenance of conserved captive populations.

In both types of lines five replicates of size $N = 20$ (10 males and 10 females) and two replicates of size $N = 100$ (50 males and 50 females) were established and maintained for 38 generations. For each of the replicate EC lines 5 additional pairs were maintained as reserves to be used in case of mating failures. If there were more than five failures (this occurred only in 1% of the cases in lines of size $N = 100$), additional progeny were obtained from randomly chosen successful vials. Thus, in these rare cases, the contribution of some couples to the next generation was four rather than two progeny. In each generation, and for both treatments, parents were maintained in their respective bottles or vials for 5 days before being discarded.

With the progeny from the wild females (generation −3) not used in the establishment of the experimental lines, 10 bottles with ~50 males and 50 females each were created and maintained, following a circular scheme to keep the population with a large effective size. These bottles represent the base population and were analyzed for fitness at generations 20 and 38.

In the case of the EC lines, where all matings were made in vials, genealogies were recorded for the whole experiment (generations −3–38), allowing the calculation of the expected

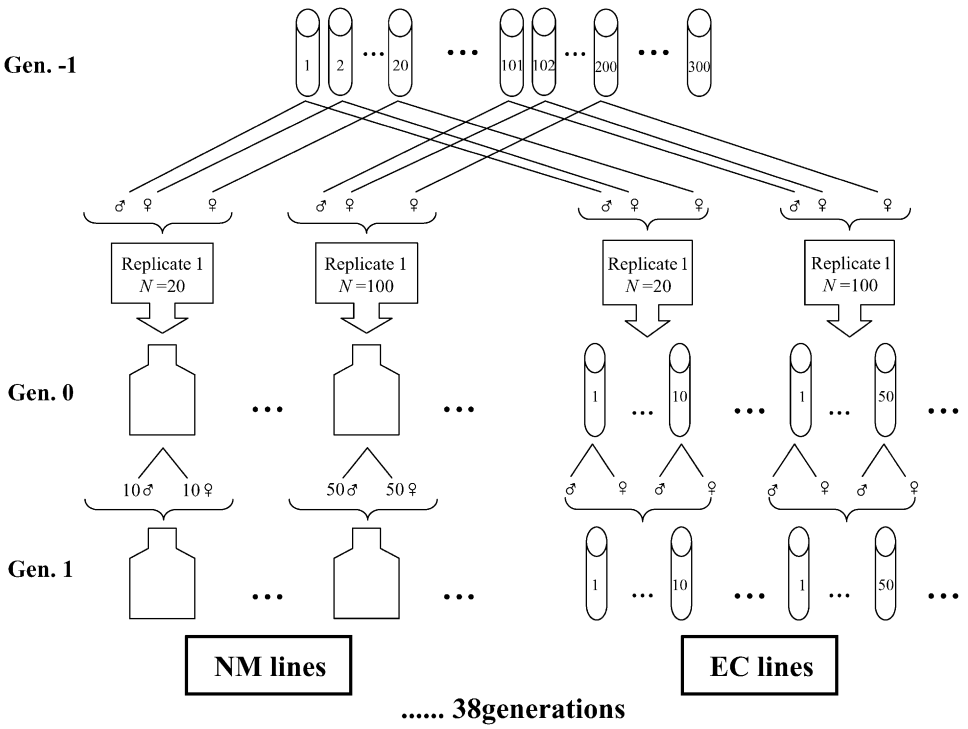

FIGURE 2.—Experimental design for the foundation and maintenance of equalization of contribution (EC) and no management (NM) lines.

inbreeding coefficient of every individual. For the NM lines pedigrees could not be recorded and the average inbreeding coefficient of each generation was predicted from the expected effective population size (see below).

Neutral genetic diversity: Neutral genetic diversity was evaluated by means of the analysis of gene and allelic diversity of microsatellite loci and of quantitative genetic variation for a quasi-neutral trait, sternopleural bristle number.

Variability for microsatellite loci: Flies from each replicate and line were stored at −20° at generations 0, 10, 20, 30, 35, and 38. DNA was extracted by the chelex method (Estoup *et al.* 1996) and a total number of 1312 individuals were analyzed for four microsatellite loci: *DROYTD3* (located in chromosome *X*; Schug *et al.* 1998), *BIB* (located at the left arm of chromosome II; Michalakis and Veuille 1996), *DMRHOb* (located at the left arm of chromosome III; Schug *et al.* 1997), and *DRONANOS* (located at the right arm of chromosome III; Goldstein and Clark 1995). PCR amplifications were carried out in a total volume of 20 μl containing 59 ng DNA, 1 μl of 20 mm each primer, 1 μl of 10 mm each deoxynucleotide, 1 μl of 50 mm $MgCl_2$, 2 μl of 10× NH_4 buffer, and 1 unit of Biotaq DNA polymerase (Bioline). PCR conditions were the following: initial denaturing step at 95° for 5 min; 5 (*BIB* and *DRONANOS*)–10 (*DROYTD3* and *DMRHOb*) cycles of 95° for 15 sec, annealing at 57° (*BIB* and *DRONANOS*) and 54° (*DROYTD3* and *DMRHOb*) for 15 sec, and extension at 72° for 15 sec; 20 (*DROYTD3* and *DMRHOb*)–30 (*BIB* and *DRONANOS*) cycles of 95° for 15 sec, annealing at 59° (*BIB* and *DRONANOS*) and 55° (*DROYTD3* and *DMRHOb*) for 15 sec, and extension at 72° for 15 sec; and a final extension at 72° for 5 min. The size of the PCR products was determined by automated fluorescent scanning detection, employing an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA) and the GENESCAN 3.7 analysis software (ABI, Columbia, MD).

Variability for bristle number: Additive genetic variance for sternopleural bristle number was evaluated at generations 0, 5, 20, and 30 by means of a single generation of artificial selection for increased and reduced bristle number. Fifty virgin males and 50 virgin females from each replicate and type of line were evaluated for the total number of bristles in the two sternopleural plates. From these, the 10 individuals of each sex with the largest (smallest) number of bristles were selected and mated at random in pairs in vials (in the case of EC lines) and *en masse* in bottles (in the case of NM lines). Fifty male and 50 female progeny were evaluated both for the upwardly and for the downwardly selected lines.

Evaluation of fitness components: *Egg-to-adult viability:* This trait was evaluated at generations 0, 5, 10, 20, 30, and 35. In each generation, 10 pairs of 5-day-old virgin individuals in each of the replicates of $N = 20$ and 50 pairs in the replicates of $N = 100$ were evaluated simultaneously. Individuals were mated as single pairs in vials (EC lines) or as groups in bottles (NM lines) and were allowed to mate for 48 hr. After this time, pairs of parents were transferred to special containers with fresh medium to which food coloring was added to visualize the eggs. Oviposition was allowed for 24 hr. From each laying, 30 eggs were transferred to a fresh vial with medium and allowed to develop into adults. The trait measured was the proportion of adults that emerged from the 30 eggs on the 13th day after the transfer, when emergence had been completed, except for very rare exceptions. When the number of eggs laid was <30 (10% of the cases), all eggs available were transferred.

Mating success: For each replicate and line, 10 vials with medium were established to simultaneously evaluate the mating success of males at generations 0, 5, 10, and 20. Two 6-day-old males of a given line and replicate were introduced in each vial along with two males and two virgin females of the same age

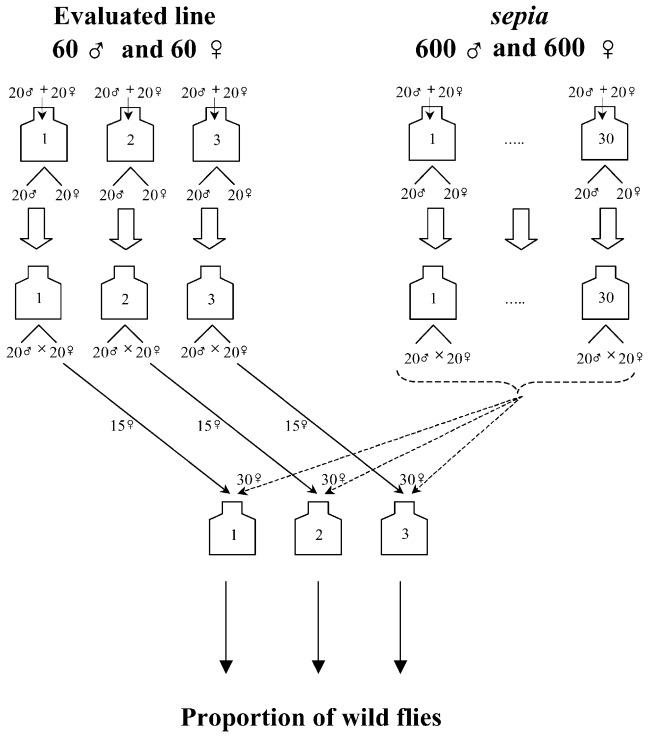

Figure 3.—Design for the estimation of global fitness in each of the experimental lines.

homozygous for the eye color recessive mutant *sepia*. These individuals belonged to an isogenic line (Caballero *et al.* 1991) maintained by full-sib mating for a large number of generations (Chavarrías *et al.* 2001) that was used as a reference line for competition. Parents were eliminated after three days and the mating success of males from the line in competition with the *sepia* males was estimated as the proportion of wild-type progeny that emerged by day 13 with respect to the total number of adults emerged.

Fitness: A global estimate of fitness for the lines and the base population was obtained by means of a competition experiment between pregnant females and their progeny from each line and those from the reference isogenic line *sepia*. This measure was carried out at generations 20 and 38 and, for that, 15 bottles with 40 individuals each (20 males and 20 females) were established (3 from the pooled replicates of $N = 20$ and 3 from the pooled replicates of $N = 100$, both for the NM and EC lines, and 3 from the pooled base population). Males and females constituting the 3 bottles for each treatment were obtained from the corresponding replicate lines in approximately equal proportions. In addition, 30 bottles also with 40 individuals each were established from the *sepia* population. The procedure followed, which was simultaneous for all types of lines, is illustrated in Figure 3. The founders of the bottles were discarded after 5 days and 20 virgin male and female progeny were obtained and mated in bottles to get again 20 virgin male and female progeny. These were then mated in bottles and, after 3 days, 15 females, presumably pregnant, were introduced in the final evaluation bottles along with 30 *sepia* females that had followed a similar procedure. The 45 females were discarded on the 5th day and from the 10th day, the number of emerging adults was counted daily for 8 (evaluation at generation 20) and 10 (evaluation at generation 38) consecutive days. The estimate of fitness is the proportion of wild-type adults that emerged with respect to the total number of adults emerged. This estimate is a multitrait

measure, including the possibility that the females might or might not be pregnant, the egg production of the pregnant females, and the egg-to-adult viability of the progeny in competition.

The *sepia* line used at generation 20 was an isogenic line. This had the advantage of being genetically uniform, providing a useful point of reference to estimate the fitness of each line. However, it had the disadvantage of having a very limited reproductive capacity and it was difficult to maintain with a large census size. Its competitive ability was very much reduced with respect to the wild flies, as was shown by the results of the analysis at generation 20. Thus, it was decided to introgress half of the genome of the base population into the *sepia* line and to use this enhanced line for the analysis of fitness at generation 38. For this, virgin females from the base population were crossed to males from the isogenic *sepia* line. The F_1 progeny was intercrossed and flies with the *sepia* phenotype were chosen in the F_2. These were then expanded for a few generations to obtain the census number (30 bottles with 40 individuals each) necessary for the fitness analysis.

Parameter estimation and statistical analyses: *Microsatellite data:* Genetic diversity for microsatellites was estimated from allele frequencies using the program GENEPOP 3.3b (Raymond and Rousset 1995), as gene diversity (expected heterozygosity, H) (Nei 1987) and as allelic diversity (n_a, number of different alleles).

Under a neutral model the ratio of gene diversities at generations t and 0 is $H_t/H_0 = (1 - F_t)$ (Falconer and Mackay 1996), where F_t is the average inbreeding coefficient at generation t. Observed gene diversities from microsatellite data, H_t, were compared with expectations, H_0 $(1 - F_t)$, using values of F_t from genealogies (EC lines) and expected effective sizes (EC and NM lines). In addition, the fit between observations and neutral expectations was tested, regressing the ratio H_t/H_0 on F_t and comparing the observed coefficient of regression and intercept with the expected values of −1 and 1, respectively. Estimates of F_t from genealogies (EC lines) were obtained, giving three times more weight to autosomal than to *X*-linked inbreeding coefficients, as microsatellite data were provided by three autosomal and one *X*-linked loci. Accordingly, estimates of F_t from the expected effective size were obtained from the expression $F_t = 1 - [(1 - 1/2N_{e,auto})^{0.75} \times (1 - 1/2N_{e,sex})^{0.25}]^t$. For EC lines, the effective population size for autosomal genes is $N_{e,auto} = 2N - 2$ (which is the expectation under equalization of contributions and avoidance of full-sib matings; Wang 1997b) and, for *X*-linked genes, $N_{e,sex} = 9N/4$ (Wright 1933; Caballero 1994, 1995). For NM lines, two predictions of $N_{e,auto}$ were assumed, N or $2N/3$, the latter corresponding to a lottery polygyny mating system (Nunney 1993). However, microsatellite data from the present experiment (see below) provided good support for $N_e \approx N$ in the NM lines, so most results are given under this assumption. For *X*-linked genes, $N_{e,sex} = 3N_{e,auto}/4$ (Wright 1933; Caballero 1994, 1995).

Bristle number data: The realized heritability (h_r^2) was calculated from the ratio of the divergence between the means for upward and downward selected lines and the sum of the selection differentials applied in both directions of selection (Falconer and Mackay 1996). The estimated additive genetic variance was obtained by multiplying the realized heritability by the corresponding phenotypic variance in the parental generation.

Fitness components data: Inbreeding depression for viability and fitness was estimated for each replicate and line as the linear regression coefficients of the mean viability or fitness (in real and logarithmic scales) on the expected inbreeding coefficient and averaged over replicates. Expected inbreeding coefficients of the EC lines were obtained from pedigrees (autosomal loci). Those for NM lines were predicted assuming an effective size $N_e = N$.

A repeated measures linear model was used to test significance between factors (type of line: EC *vs.* NM) and census size ($N = 100$ *vs.* $N = 20$) for all neutral and fitness parameters. Statistical comparisons between means were made by *t*-tests for related values, using all generations except generation zero, and by Mann-Whitney tests. The evolution of all parameters over generations was also described by linear and quadratic regressions. All analyses involving ratios (gene diversity, viability, mating success, and fitness) were done both for untransformed data and for arcsine square-root-transformed data, but the latter data gave very similar results to the former in all tests and are not presented. Pearson correlations were used to quantify the relationship among parameters. All analyses were done using SPSS 11.5.1.

RESULTS

Evaluation of the genetic diversity: *Microsatellite variation:* Table 1 shows the gene diversity and the number of alleles per locus averaged over the four microsatellite loci studied and over all replicates. A repeated measures linear model showed significance for the type of line (EC *vs.* NM; $P = 0.026$) and for the census size ($N = 100$ *vs.* $N = 20$; $P = 0.0002$), but not for the interaction between them ($P = 0.628$). A *t*-test for related values using generations 10–38 showed that heterozygosity declined significantly more in the NM lines than in the EC lines both for $N = 100$ ($P = 0.001$) and for $N = 20$ ($P = 0.0001$). Thus, the linear regression of gene diversity on generation number was -0.0009 ± 0.0012 (average linear drop of 7% from the initial mean) in the EC $N = 100$ lines *vs.* -0.0030 ± 0.0010 (20% drop) in the NM $N = 100$ lines and -0.0050 ± 0.0013 (36% drop) in the EC $N = 20$ lines *vs.* -0.0068 ± 0.0012 (47% drop) in the NM $N = 20$ lines. Further, the number of alleles dropped significantly more in the NM lines than in the EC lines both for $N = 100$ (*t*-test for related values, $P = 0.007$) and for $N = 20$ ($P = 0.002$) (repeated measures linear model: $P = 0.001$ for type of line, $P = 0.00001$ for census size, and $P = 0.389$ for the interaction between these two factors).

Table 1 also shows the predicted declines in gene diversity expected from inbreeding coefficients obtained from genealogies (EC lines), as well as assuming the expected effective population size for each type of line. For the EC lines, predictions from genealogical inbreeding coefficients were almost identical to the expectations from predicted effective sizes, suggesting that the procedure followed for the equalization of family sizes was the intended one. Overall, the agreement between observed gene diversities and predictions from expected effective population sizes was reasonably good, except for particular generations. The assumption of an effective size of $2N/3$ for autosomal loci ($N/2$ for *X*-linked loci) in the NM lines implied a substantially worse agreement with observations from generation 30 onward (data not shown). Thus, an effective size of $N_e = N$ is assumed for NM lines henceforth.

TABLE 1

Gene diversity (expected heterozygosity) and number of alleles per locus (± standard errors) averaged over microsatellite markers and replicates, and predicted gene diversities

		Generation					
	Line	0	10	20	30	35	38
				Gene diversity			
$N = 100$	EC	0.55 ± 0.01	0.66 ± 0.04	0.59 ± 0.03	0.57 ± 0.00	0.49 ± 0.00	0.61 ± 0.04
		0.55	*0.54[a] ± 0.01*	*0.52[a] ± 0.01*	*0.51[a] ± 0.01*	*0.50[a] ± 0.01*	*0.49[a] ± 0.01*
		0.55	*0.54[b]*	*0.52[b]*	*0.51[b]*	*0.50[b]*	*0.50[b]*
	NM	0.56 ± 0.03	0.54 ± 0.04	0.44 ± 0.04	0.48 ± 0.02	0.42 ± 0.01	0.47 ± 0.03
		0.56	*0.53[b]*	*0.50[b]*	*0.48[b]*	*0.46[b]*	*0.46[b]*
$N = 20$	EC	0.52 ± 0.03	0.49 ± 0.01	0.32 ± 0.07	0.37 ± 0.04	0.35 ± 0.03	0.32 ± 0.04
		0.52	*0.48[a] ± 0.03*	*0.42[a] ± 0.03*	*0.37[a] ± 0.03*	*0.34[a] ± 0.02*	*0.33[a] ± 0.02*
		0.52	*0.46[b]*	*0.40[b]*	*0.35[b]*	*0.33[b]*	*0.32[b]*
	NM	0.56 ± 0.02	0.39 ± 0.04	0.23 ± 0.05	0.29 ± 0.05	0.27 ± 0.03	0.26 ± 0.03
		0.56	*0.43[b]*	*0.32[b]*	*0.25[b]*	*0.21[b]*	*0.20[b]*
				Allelic diversity			
$N = 100$	EC	4.88 ± 0.38	4.50 ± 0.25	3.50 ± 0.00	3.88 ± 0.13	3.25 ± 0.00	3.25 ± 0.25
	NM	5.00 ± 0.25	3.13 ± 0.38	3.00 ± 0.00	2.50 ± 0.25	2.38 ± 0.13	2.63 ± 0.13
$N = 20$	EC	3.75 ± 0.14	2.80 ± 0.09	2.05 ± 0.24	2.25 ± 0.21	2.55 ± 0.27	2.25 ± 0.24
	NM	3.55 ± 0.15	2.10 ± 0.15	1.70 ± 0.18	1.80 ± 0.09	1.80 ± 0.12	1.65 ± 0.06

Predicted gene diversities are in italics. *N*, population size; EC, equalization of contributions; NM, no management (free contributions).
[a] Prediction of the drop in gene diversity for EC lines using the inbreeding coefficients obtained from genealogies.
[b] Prediction of the drop in gene diversity using the expected effective population size for EC lines ($2N-2$ for autosomal loci and $9N/4$ for *X*-linked loci) and NM lines (N for autosomal loci and $3N/4$ for *X*-linked loci).

The relationship between the ratio of gene diversities to the initial value (H_t/H_0) and the inbreeding level is shown in Figure 4A for each type of line, replicate, and generation. The regression coefficient, -0.928 ± 0.113, and the intercept, 0.993 ± 0.040, were very close to their predictions under a neutral model (-1 and 1, respectively).

Bristle number variation: The estimates of realized heritability for sternopleural bristle number averaged over replicates are shown in Table 2. A repeated measures linear model showed significance for the type of line ($P = 0.037$), for the census size ($P = 0.019$), and for their interaction ($P = 0.023$). Values of heritability were significantly lower for the NM lines than for the EC lines for $N = 20$ (*t*-test for related values, $P = 0.001$), but not for $N = 100$ ($P = 0.919$).

Figure 4B shows the relationship between the ratio of additive genetic variances to the initial value (V_{A_t}/V_{A_0}) and the inbreeding level. The regression coefficient, -1.402 ± 0.523, and the intercept, 1.070 ± 0.145, had large standard errors but were significantly different from zero and nonsignificantly different from their neutral predictions.

Evaluation of fitness traits: *Egg-to-adult viability:* Figure 5A shows the evolution of the average viability of the NM and the EC replicate lines across generations. The viability of the NM lines with $N = 100$ was clearly lower than that of the others at generation 0, and this difference was somewhat apparent throughout the experiment. The reason is likely to be environmental. Because evaluated virgin flies were taken directly from the experimental bottles (NM lines) and vials (EC lines), it is possible that the viability of progeny from females grown in bottles (particularly those with 100 flies) was lower than that from females grown in low-density media (vials and bottles with 20 flies). Thus, the comparison between lines should be done in terms of the relative decline in viability. Accordingly, viabilities for each type of line and replicate were analyzed as deviations from the corresponding initial means. Average deviations are presented in Figure 5B, suggesting a consistent decline of viability for all types of lines. This decline was reasonably linear (fit to linear regressions $r^2 = 0.51$, averaging over all replicated lines), and the fit to quadratic regressions was only slightly improved (average $r^2 = 0.63$). For replicates with $N = 100$, the average linear declines were -0.0044 ± 0.0020 and -0.0042 ± 0.0001 for the EC and the NM lines, respectively, corresponding to overall proportional declines of 23 and 32% of the initial mean. For replicates with $N = 20$, the average linear declines were -0.0065 ± 0.0007 and -0.0075 ± 0.0012 for the EC and the NM lines, respectively, corresponding to overall proportional declines of 32 and 40% of the initial mean. A repeated measures linear model showed significance for census size ($P = 0.038$), but not for type of line ($P = 0.924$) or for the

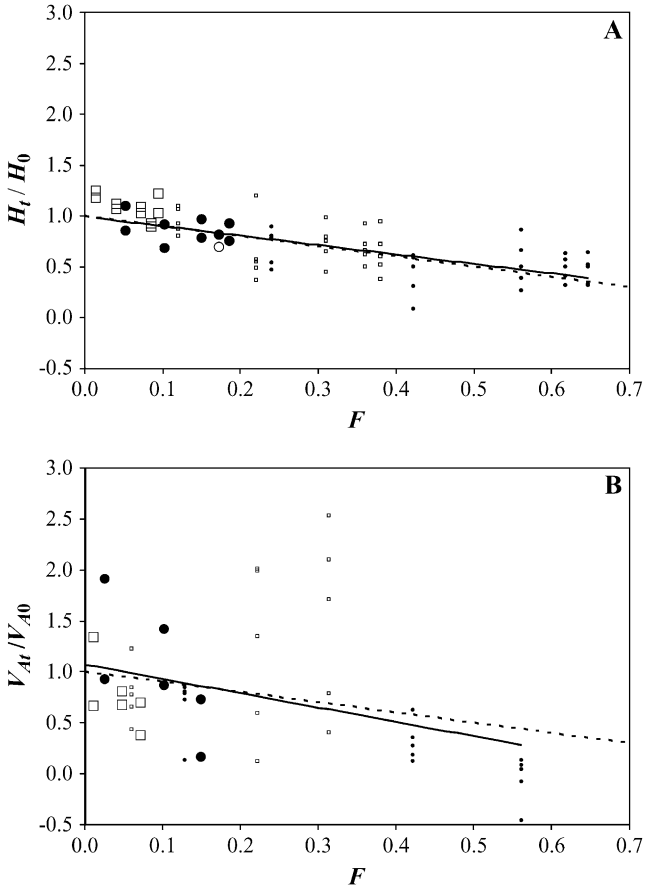

FIGURE 4.—Regression of the ratio of gene diversities (expected heterozygosity, H) for microsatellites and averaged additive genetic variances (V_A) for sternopleural bristles, at generations t and 0, on inbreeding coefficient. This latter estimate was obtained from genealogies for the EC lines and by using an expected effective size of $N_e = N$ (autosomal) and $3N/4$ (X-linked) for the NM lines. In both cases, three times more weight was given to autosomal than to X-linked estimates. Circles, NM lines; squares, EC lines. Large symbols, $N = 100$; small symbols, $N = 20$. The dashed line represents the neutral prediction (intercept 1 and slope −1).

interaction ($P = 0.558$). In addition, a t-test for related values did not show significant differences between the EC and the NM lines, either for $N = 100$ ($P = 0.554$) or for $N = 20$ ($P = 0.127$). Similar results were obtained with logarithmic and arcsine square-root-transformed data.

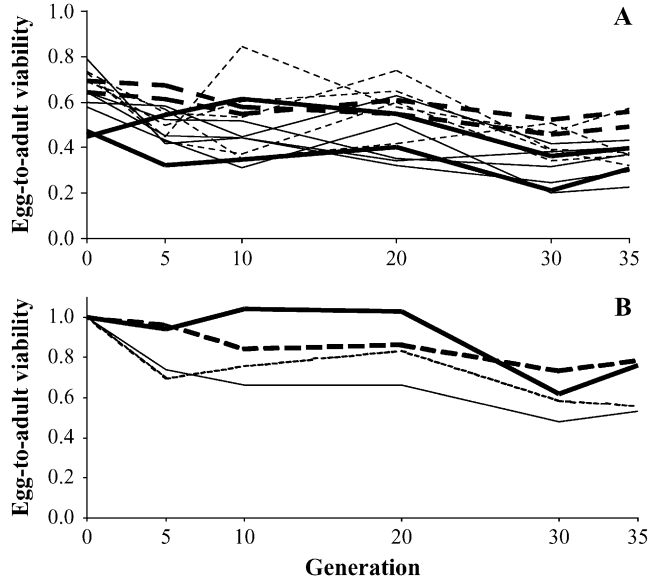

FIGURE 5.—Average egg-to-adult viability for equalization of contributions (broken) and no management (continuous) lines with population sizes $N = 100$ (thick lines) and 20 (thin lines). (A) Values for individual replicates. (B) Average over replicates for each type of line deviated from the corresponding initial mean.

The average inbreeding coefficients of the EC lines at generation 38 obtained from pedigrees (autosomal loci) were 0.09 ± 0.00 and 0.37 ± 0.02, for lines with $N = 100$ and 20, respectively, in good agreement with those predicted assuming an effective size $N_e = 2N - 2$ (0.09 and 0.40). Likewise, the expected average inbreeding coefficients of the NM lines at generation 38 assuming an effective size $N_e = N$ were 0.17 and 0.62, respectively.

The estimates of inbreeding depression per 1% increase in inbreeding averaged over replicates are presented in Table 3. As expected from the less effective purging of deleterious alleles in the EC lines, inbreeding depression estimates were larger for the EC lines than for the NM lines for both population sizes, although not significantly so. This result was also observed for fitness evaluations (Table 3; see below). The larger estimated inbreeding depression for lines of $N = 100$ *vs.* lines of $N = 20$ is likely to be caused by the much higher

TABLE 2

Realized heritability for sternopleural bristle number (± standard errors) averaged over replicates

		Generation			
	Line	0	5	20	30
$N = 100$	EC	0.62 ± 0.01	0.45 ± 0.07	0.57 ± 0.10	0.54 ± 0.10
	NM	0.65 ± 0.06	0.64 ± 0.05	0.70 ± 0.27	0.27 ± 0.17
$N = 20$	EC	0.59 ± 0.10	0.42 ± 0.03	0.54 ± 0.16	0.62 ± 0.14
	NM	0.49 ± 0.05	0.30 ± 0.08	0.15 ± 0.04	−0.01 ± 0.04

N, population size; EC, equalization of contributions; NM, no management (free contributions).

TABLE 3

Inbreeding depression per 1% increase in inbreeding (± standard errors) averaged over replicates

	Line	Viability	Fitness (gen 20)	Fitness (gen 38)
$N = 100$	EC	1.59 ± 0.74	1.13 ± 0.50	1.51 ± 0.15
	NM	0.91 ± 0.04	0.63 ± 0.31	1.19 ± 0.24
$N = 20$	EC	0.60 ± 0.08	0.61 ± 0.34	0.67 ± 0.17
	NM	0.47 ± 0.08	0.37 ± 0.04	0.17 ± 0.09

N, population size; EC, equalization of contributions; NM, no management (free contributions).

inbreeding levels reached in the latter lines. For increasing levels of inbreeding, purging of deleterious alleles is expected to restrain the rate of viability decline (see, *e.g.*, García *et al.* 1994). In fact, the magnitude of the inbreeding depression for lines with $N = 20$ was similar to that for lines of $N = 100$ when only the first stages of the experiment (up to an inbreeding of ~0.1) were considered. Thus, the initial estimated inbreeding depression values for viability were 1.72 ± 1.41 and 1.42 ± 0.49 for the EC and the NM lines of $N = 20$, respectively.

Mating success: The average mating success of males from the experimental lines relative to males of the *sepia* reference line did not suffer any appreciable changes across generations (averages of 0.97, 0.91, 0.90, and 0.86 for generation 0 and 0.94, 0.95, 0.92, and 0.92 for generation 20, for lines EC $N = 100$, NM $N = 100$, EC $N = 20$, and NM $N = 20$, respectively). No significant differences between EC and NM lines were detected (*t*-test for related values, $P = 0.423$ for $N = 100$ and $P = 0.336$ for $N = 20$; repeated measures linear model analysis, $P = 0.483$ for type of line factor). Thus, no further evaluations were made.

Fitness: The proportion of wild-type flies in each of the three bottles that were used for the experimental evaluations of global fitness is shown in Figure 6. The drastic difference in scale between both evaluations is due to the fact that the reference line used for competition was the original isogenic line at generation 20, whereas a cross between this line and the base population was used at generation 38. Thus, most emerging progeny (from a total of 301 ± 84 flies per bottle, on average) were wild type in the generation 20 analysis because the experimental wild-type flies outperformed the *sepia* isogenic flies. In contrast, a more equal competition and with much higher densities (1320 ± 27 flies per bottle, on average) occurred at generation 38. Relative fitness at generation 38 for the base population was 0.37 ± 0.02, nonsignificantly different from the expectation (0.33) if all of the genome in the derived *sepia* line were from the base population or if most deleterious genes in the original *sepia* line were recessive and were masked by the base population alleles.

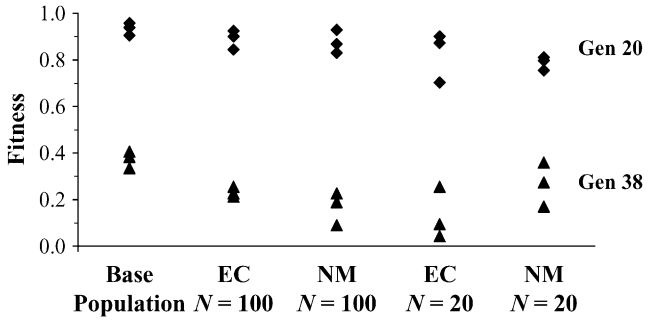

Figure 6.—Estimates of global fitness for equalization of contributions (EC) and no management (NM) lines with population sizes $N = 100$ and 20, as well as for the base population at generations 20 and 38.

TABLE 4

Pearson correlations between different parameters analyzed

	Mating success	H	n_a	h^2
Viability	−0.21	0.38*	0.48*	0.32*
Mating success		−0.08	−0.03	0.14
H			0.84*	0.43*
n_a				0.43*

Viability, egg-to-adult viability; H, gene diversity (expected heterozygosity) for microsatellite markers; n_a, average number of alleles per locus for microsatellite markers; h^2, average realized heritability for sternopleural bristle number. *Significantly different from zero at a 5% level. Number of pairs used for correlations: $n = 84$ for correlations between H and n_a; $n = 70$ for correlations between viability and H and n_a; $n = 56$ for correlations between viability and h^2; $n = 42$ for correlations between h^2 or mating success and the other parameters.

Fitness declined significantly with respect to the base population estimate for the EC and the NM lines of size $N = 20$ (Mann-Whitney test, $P = 0.05$) at generation 20 and for the EC lines ($N = 100$ and 20) and the NM lines ($N = 100$) at generation 38. No significant differences, however, were found either between the fitnesses of EC and NM lines or between census sizes (repeated measures linear model: $P = 0.822$ for type of line factor, $P = 0.298$ for census size factor, and $P = 0.230$ for their interaction). Inbreeding depression estimates per 1% increase in inbreeding averaged over replicates are shown in Table 3. The magnitudes of inbreeding depression were of the same order as those shown previously for viability. Again, a trend was observed for EC lines showing lower rates of inbreeding depression than NM lines for both population sizes, although differences were not significant.

Correlation between neutral and adaptive variation: The Pearson correlations between the different parameters estimated, including all data, are shown in Table 4. Egg-to-adult viability showed a significant positive correlation with all estimates of neutral variation (microsatellite and bristle number). Mating success, however,

was uncorrelated with all other parameters. The realized heritability for sternopleural bristle number showed a significant correlation with microsatellite variation.

DISCUSSION

Although equalization of parental contributions is a widely recognized method to keep genetic diversity in conservation programs (Wright 1938; Gowe *et al.* 1959; Wang 1997a; Sánchez *et al.* 2003), it has been suggested that the reduced purging of deleterious mutations implied by the lack of selection between families may have negative consequences on the reproductive capacity of the populations (Lange 1981; Couvet and Ronfort 1994; Schoen *et al.* 1998). Recent theoretical studies comparing equalization of contributions and free contributions (Fernández and Caballero 2001a,b; Theodorou and Couvet 2003) concluded that equalization of contributions may lead to a lower fitness than that for schemes with free contributions but (1) only to a low extent, (2) after a relatively large number of generations (>10–20), (3) for relatively large population sizes (*e.g.*, $N > 30$ after 20 generations), and (4) for a magnitude that depends on the selection model assumed for the lines with free contributions. Regarding point 4, Fernández and Caballero (2001a) assumed two models of selection for free contribution lines. In a random-mating model, polygamous matings were considered and the fecundity of each individual was assumed to be independent of that of its partner. In a random-paired mating model, monogamous matings were considered instead, where the fecundity of the couple is assumed to be the average fecundity of both parents. Because in this latter model the intensity of selection for fecundity is reduced relative to that of the former model (the between-family component is reduced to a half), less difference is expected between lines with equalization of contributions and lines with free contributions under the second than under the first model. Fernández and Caballero (2001a), however, erroneously ascribed this difference between the random and random-paired models to the monogamous *vs.* polygamous mating system in itself, rather than to the different models of fecundity selection being assumed.

Our results, involving relatively large population sizes, a long management period, and full competition in free contribution lines, do not provide evidence to suggest that equalization of contributions entails an important disadvantage on the reproductive capacity of conserved populations in comparison with no management procedures. It should be noted, however, that because the selection intensity against deleterious alleles is reduced in the EC lines, less purging of these deleterious alleles occurs in the EC lines relative to that in the NM lines. If deleterious alleles are partially recessive (usually the case), one would expect more inbreeding depression per unit of inbreeding under EC than under NM lines. This can be confirmed from simulation studies. For example, in the model of fecundity and viability shown in Fernández and Caballero's (2001a) Figure 1d, the inbreeding depression per 1% of increase in inbreeding up to generation 40 is 4.9 times larger for EC $N = 100$ lines than for NM $N = 100$ lines (2.3 times larger for the case of $N = 25$) (A. Caballero, unpublished results). This trend is clearly observed in the experimental results (Table 3), with the inbreeding depression of EC lines being always larger than that of NM lines, although nonsignificantly so and not to a large extent (except for fitness at generation 38 and $N = 20$). However, because EC lines generate less cumulative inbreeding than NM lines for the same period of time, this effect counterbalances the lower selection intensity (higher inbreeding depression) in EC lines, yielding the overall result that EC lines do not show a lower viability and fitness than NM lines.

It is apparent from the above results that the method of equalization of contributions involves the maintenance of a higher overall genetic diversity with two effects of opposite sign. On the one hand, a higher genetic diversity implies a higher adaptive potential but, on the other hand, also a larger deleterious segregation load, which may be expressed as inbreeding depression. The balance between these two factors will depend on the future demographic changes of the population and the opportunities for adaptation to new environmental challenges. If the population suffers from severe bottlenecks, either in captivity or after reintroduction in the wild, the higher concealed deleterious variation occurring with equalization of contributions can be expressed as inbreeding depression, with negative consequences on the population viability. In contrast, the extra genetic variation can be exploited by the population for adaptation to new environmental conditions in the wild. Therefore, provided that extreme bottlenecks are avoided, equalization of contributions is likely to imply a benefit rather than a disadvantage.

Although equalization of family sizes reduces selection among families to a minimum, some selection of this type inevitably occurs, as there are a certain number of unfertile matings. During the experiment, a few additional pairs were always maintained in the EC lines to be used in case of mating failures. The number of substitutions was generally small but increased steadily over generations, suggesting some selection between lines. Thus, the proportion of substitutions increased from 5% at generation 0 to 8% at generation 38 in the lines of $N = 100$ and from 9% at generation 0 to 20% at generation 38 in the lines of $N = 20$.

Egg-to-adult viability showed a substantial decline over 35 generations, between 23 and 40% of the initial mean, depending on the type of line and census size. These values are of the same order as those obtained for a similar period of time in simulations using a model of deleterious mutations with large mutation rate and low mean effect of mutations (see Fernández and

Caballero 2001a). Simulation models with low mutation rate did not show such appreciable declines in viability, but it should be noted that the simulation results of Fernández and Caballero (2001a) and all other theoretical studies on this topic (Schoen *et al.* 1998; Fernández and Caballero 2001b; Theodorou and Couvet 2003) are based on the assumption of deleterious mutations in the base population segregating at mutation-selection balance frequencies. Thus, although the decline in viability observed in this experiment is compatible with mutational models of large mutation rate and small average mutational effect under mutation-selection balance, other models with a low rate of mutations but involving a few genes under balancing selection (Charlesworth and Hughes 1999; Fernández *et al.* 2005) could probably not be discarded.

The final objective of a conservation program may be the reintroduction into the wild of the captive population (Frankham *et al.* 2000). If this is the case, two important aspects should be noted. First, because equalization of family sizes is supposed to minimize selection, a positive consequence is that adaptation to captivity is also reduced (Allendorf 1993; Frankham *et al.* 2000). Second, the relaxation of selection that occurs under the benign conditions of captivity may have the consequence that the selection coefficients against deleterious mutations are overly reduced. Fernández and Caballero (2001a) theoretically investigated the impact of a reduction of up to a tenth in the magnitude of the selection coefficients of mutations under captive conditions during 50 generations. The population was assumed to be thereafter released to the wild, so that selection coefficients recovered their original values. Because the relaxation of selection under captive conditions implies that fixation of mutations is basically driven by genetic drift, and the equalization of contributions method increases the effective population size, fixation of mutations was lower for this method than for the method with random contributions. Thus, reintroduced populations maintained by equalization of contributions would be expected to have higher fitness than reintroduced populations maintained with random contributions. A similar conclusion has been reached by Theodorou and Couvet (2004), theoretically analyzing the increase in fitness of a wild population subject to periodic reintroductions from a captive breeding one. In an empirical test with *Musca domestica*, Meffert *et al.* (2005) compared the performance under experimentally simulated wild conditions of lines previously maintained under a maximum avoidance of inbreeding scheme (equalization of family sizes and avoidance of inbred matings) and under a free-mating scheme where individuals contribute offspring in proportion to their estimated fecundity and fertility. The maximum avoidance of inbreeding scheme resulted in a significantly higher fitness under wild conditions.

It is possible that some adaptation to the laboratory medium occurred during the experiment, particularly since our base population was a recently caught one. As the time to fixation of advantageous mutations is expected to be considerably longer under equalization of family sizes than under free contributions (Caballero *et al.* 1996), any possible adaptation should be faster in the NM lines than in the EC lines (see Frankham *et al.* 2000). According to this, we would expect a lower reduction in viability and fitness over generations in the NM lines than in the EC lines. This would increase the expected superiority of NM over EC lines in the long term. The observed lack of such a superiority, however, suggests that the putative larger adaptation to the medium of the NM lines was not very pronounced.

Estimates of fitness were carried out in bottles, involving competition between individuals of the tested line and others from a marker reference population. It may be argued that NM lines, which were maintained throughout the experiment in bottles, could be better adapted to these competitive conditions than EC lines, in which individuals were maintained in single pairs in vials. In this latter case, full-siblings are raised together without competition from unrelated individuals, reducing selection for competitive ability. Thus, EC males evolve lower semen toxicity and spend less time harassing females, and EC females evolve increasing susceptibility to seminal fluids (Holland and Rice 1999). In fact, as shown by Woodworth *et al.* (2002), lines of *D. melanogaster* maintained with equalization of contributions drastically declined in fitness when evaluated in competition with marker flies in bottles. In contrast, a control population maintained in bottles for many generations increased in fitness when this was measured in competitive bottles, but decreased in fitness when evaluated in terms of the number of progeny from single pairs in vials. If this effect occurred in our lines we would expect a higher fitness performance for the NM lines than for the EC lines, particularly for the largest census size. However, for our lines of $N = 100$, the average fitness performance of the EC lines was greater (although nonsignificantly so) than that for the NM lines, at both generations 20 and 38 (Figure 6). This result, therefore, does not support a substantial effect of adaptation.

The evaluation for viability was performed in vials, so a similar argument could be applied, this time favoring the EC lines, where individuals were maintained in vials throughout the experiment. However, the estimate of viability was the percentage of adults emerging from 30 eggs laid in a container, irrespective of the total number of eggs laid and the mating of individuals, which took place in vials (EC lines) or bottles (NM lines). Therefore, adaptation to competitive conditions in bottles or to noncompetitive conditions in vials is not expected to largely affect the estimates of viability.

Finally, the base population (BP) was maintained in a medium slightly poorer than that of the maintenance of the EC and NM lines. Fitness was evaluated on the richer

medium at generation 20 and on the poorer one at generation 38. In both evaluations the BP outperformed the NM and EC lines (Figure 6), as expected. Nevertheless, the relevant comparison is the relative difference in fitness between the NM and EC lines, and these were both maintained in the richer medium.

As expected, EC lines maintained greater heterozygosity and allelic richness for microsatellite markers and greater heritability for sternopleural bristle number. Gene diversity for microsatellites and additive genetic variance for sternopleural bristle number declined with inbreeding as expected from a strictly neutral prediction. The regression coefficient of the decline in genetic variation on the level of inbreeding was -0.928 ± 0.113 for microsatellites and -1.402 ± 0.523 for sternopleural bristle number, both nonsignificantly different from their neutral prediction (-1). These are somewhat larger than the corresponding regressions observed by Gilligan *et al.* (2005) for allozymes (-0.786 ± 0.100) and for abdominal (-0.668 ± 0.136) and sternopleural (-0.581 ± 0.105) bristle numbers. The reason may be that the range of inbreeding coefficients investigated by Gilligan *et al.* (2005) (up to $F \approx 1$) was larger than that in the present experiment (up to $F \approx 0.6$), and a retention of variation for highly inbred lines has been reported (see Gilligan *et al.* 2005).

Significant correlations were found between realized heritability for bristle number and gene and allelic diversity for microsatellites. More importantly, significant correlations were also found between egg-to-adult viability and microsatellite gene diversity (0.38), allelic diversity (0.48), and heritability for bristle number (0.32). Although these correlations are substantial and significant, the absolute values are not very large, in agreement with recent metaanalyses suggesting a generally low correlation between molecular and quantitative trait variability (Merilä and Crnokrak 2001; Reed and Frankham 2001; Mckay and Latta 2002; see also Gilligan *et al.* 2005). However, the fact that equalization of contributions is able to maintain larger levels of variation, and that this correlates with fitness traits, suggests that the method can be safely used as a conservation rule.

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